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A Colorimetric Assay for the Determination of Acid Nucleoside Triphosphatase Activity

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Summary: A photometric method for the determination of the acid nucleoside triphosphatase (EC 3.6.1.–) is described, in which inorganic phosphate is liberated from ATP or other nucleoside triphosphates. Colorimetric determination of liberated phosphate is based on the formation of a green complex of phosphomolybdate and malachite green hydrochloride. Optimal test conditions were evaluated as well as the sample preparation. The enzyme activities measured in 100 normal human sera are in the range of 0.5 to 9.0 U/l with an average of 4.0 U/l for men and 3.8 U/l for women.

Introduction

Recently we reported preliminary studies on a novel enzyme with an unusual pH optimum of 3.0 (1). This enzyme, acid nucleoside triphosphatase (EC 3.6.1.–), has been partly purified and characterised. The enzyme hydrolyses all nucleoside triphosphates only to their corresponding diphosphates and inorganic phosphate (2). Because of its putative lysosomal origin we have chosen myocardial infarction as a model of wound repair to investigate some aspects of its biological function. Enzymatic activity was maximally elevated six weeks after myocardial infarction (3), indicating that the enzyme did not originate from the myocardial tissue itself but probably from activated macrophages, which infiltrate the scar at that time (4).

The activity of the enzyme has so far only been measured by using radiolabeled nucleoside triphosphates as a substrate (1, 2), a method which is specific, but expensive and inconvenient for routine laboratory use. The assay presented here is based on a colorimetric determination of inorganic phosphate released by the acid nucleoside triphosphatase, using a sensitive method developed by *Lanzetta* et al. (5) and adapted to the actual assay conditions. In the present assay, phosphate forms a complex with ammonium molybdate and malachite green hydrochloride. The absorbance of the resulting green complex can be estimated at 640–660 nm.

Materials and Methods

Chemicals

All tritium-labelled nucleotides were obtained from Amersham-Buchler, unlabelled nucleotides from Boehringer-Mannheim. Malachite Green and dithiothreitol were purchased from Sigma Chemical Co., Rheomacrodex 10% was from Schiwa GmbH, Glandorf. All other chemicals were supplied by E. Merck, Darmstadt, in the highest quality grade commercially available.

Solutions

Unless otherwise indicated distilled water was used in the preparation of all solutions.

Dialysis buffer: 150 mmol/l NaCl, 10 mmol/l Tris-HCl pH 7.5, Rheomacrodex 10%, volume fraction 0.25.

Reaction buffer: The following three stock solutions,
(I) 100 mmol/l citric acid – NaOH, pH 3.0,
(II) 40 mmol/l EDTA, disodium salt,
(III) 40 mmol/l dithiotreitol,
were mixed in a ratio of 1 + 1 + 1.

Colour reagent: Mix one part of 42 g/l ammonium molybdate in 4 mol/l HCl with 3 parts of 0.45 g/l Malachite Green, stir the mixture for at least 20 min, then filter through a Whatman No. 5 filter paper. The ready-to-use reagent should not be stored for more than two weeks.

Stabilizer: 340 g/l sodium citrate.

Stop reagent: 250 g/l trichloroacetic acid.

P_i standards: Na_2HPO_4 in the range 0–250 µmol/l (0–50 µmol/l final).

Substrate solution: Nucleoside triphosphate, 1.25 mmol/l (250 µmol/l final). Store at −20 °C.

Sample preparation: 250–500 µl of serum are intensively dialysed for 12–24 hours in micro-collodion-bags (Sartorius GmbH, Göttingen) at 4 °C against the dialysis buffer.

Nucleoside triphosphatase test

Samples and reagents are pipetted according to the scheme in table 1. The total volume of the assay is 250 µl. After incubation for 30 min at 30 °C, stop reagent (50 µl) is added, the mixture vortexed, then centrifuged for 3 min (8000 min^{-1}) to pellet the protein. Supernatant (200 µl) is transferred to a new vial, followed by 1 ml of colour reagent. After 1 min of colour development, 100 µl of stabilizer are added and mixed thoroughly. Absorbance is determined at 650 nm, using water as the blank. The colour is stable for at least 4 hours.

The substrate blank and the serum blank are both subtracted from the sample values and the resulting value is referred to the P_i standard curve. One international unit (U) represents the production of 1 µmol P_i per minute.

Radioisotopic nucleoside triphosphatase assay

This assay, developed by *Dahlmann* et al. (1), uses radiolabeled nucleoside triphosphate as substrate. After incubation, nucleoside mono-, di- and triphosphates are separated by thin-layer chromatography, and radioactivity is measured by liquid scintillation counting.

Results

Dialysis

Because of the high P_i concentration in human serum (1–2 mmol/l), this metabolite must be removed by dialysis to about 1/1000 of the physiological concentration. In order to minimise volume changes of the samples during dialysis, an osmoticum should be added to the dialysis buffer. We chose a dextran solution in a final concentration of 2.5%. This dialysis buffer has the average osmolarity of human serum. Serum blanks after extensive dialysis showed an absorbance ($\bar{x} \pm s$) of 0.01 ± 0.002 making spot checks of 2 or 3 serum blanks sufficient for an entire series.

Standard curves

Since proteins in serum are capable of binding ions, we investigated the effect of serum protein on the recovery of inorganic phosphate. P_i standards in a final concentration of 0–50 µmol/l were estimated in assay mixtures containing either buffer, serum, substrate, or combinations of these; there were no significant differences in P_i recovery between them. Therefore, serum seems to have no matrix effect on P_i recovery, and the P_i standard curve can be determined in reaction buffer without the addition of protein. An absorbance of 1.0 corresponds to a P_i concentration of 60–80 µmol/l.

Substrate stability

To distinguish between enzymatic turnover and unspecific hydrolysis, the stability of different substrates was tested at various temperatures and pH values with a substrate concentration of 250 µmol/l. For these experiments background values of the substrates were not subtracted. A non-enzymatic hydrolysis value of 5% corresponds to an absorbance of 0.15. The stability of 2′-deoxythymine triphosphate (dTTP) was monitored at four different temperatures at pH 3.0. The maximal hydrolysis rate was found to be 0.5%, measured over a period of 30 minutes at 37 °C, whereas at 0 °C no hydrolysis was observed (fig. 1). An incubation temperature of 30 °C is a compromise between the optimal temperature of 43 °C for the enzymic activity (2) and a temperature that gives a low substrate blank value.

Since the assay is conducted at pH 3.0 and stopped by the addition of trichloroacetic acid, the effect of pH on the substrate stability was assessed. The non-enzymatic hydrolysis rate of 2′-deoxyadenosine triphosphate (dATP) and dTTP was measured at pH 1.0 and pH 3.0 for 60 min at 30 °C (fig. 2). The results indicate that, at pH 3.0, both dTTP and dATP were stable with a rate of hydrolysis of less than 0.5% within one hour. However, the hydrolysis rate increased significantly at pH 1.0 with a rate of 2% for dATP and 3% for dTTP. Samples should therefore be kept on ice after the addition of stop reagent, and absorbance determined within 30 minutes.

Tab. 1. Pipetting scheme for nucleoside triphosphatase test (volumes in µl).

	Water blank	Substrate blank	Serum blanks	Samples (serum)	Standard curve
Reaction buffer	150	150	150	150	150
Water	100	50	50	–	50
Serum	–	–	50	50	–
Substrate solution	–	50	–	50	–
P_i standards	–	–	–	–	50

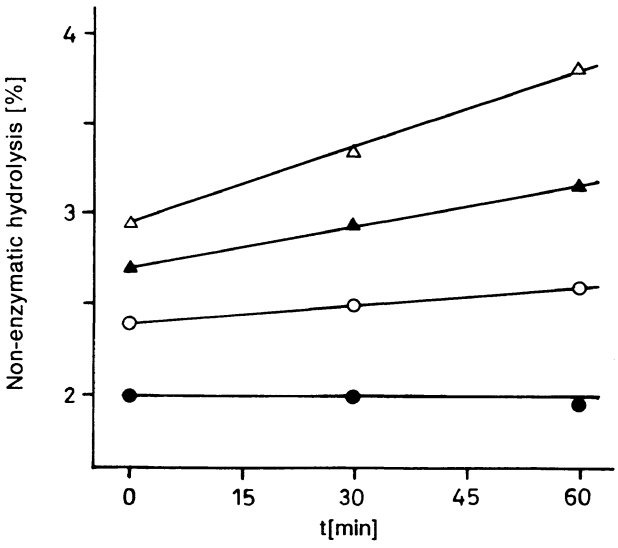

Fig. 1. Non-enzymatic hydrolysis of dTTP was measured for 60 min at pH 3.0 and four different temperatures: 0 °C (●), 20 °C (○), 30 °C (▲), and 37 °C (△).

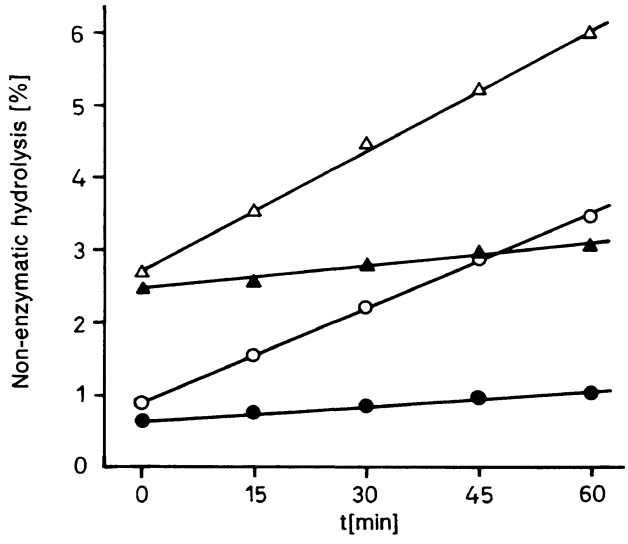

Fig. 2. Non-enzymatic hydrolysis of dATP (●, ○) and dTTP (▲, △) was measured at pH 3.0 (●, ▲) and pH 1.0 (○, △) for 60 min at 30 °C.

Tab. 2. Non-enzymatic hydrolysis of different substrates (250 µmol/l) under standard assay conditions (30 min at 30 °C and pH 3.0)

Substrate	ATP	dATP	dTTP	dCTP	dGTP	dUTP
Hydrolysis (%)	0.15	0.44	2.64	2.90	2.42	2.82

To compare the stability of different substrates, non-enzymatic hydrolysis was measured for 30 min at 30 °C and pH 3.0 at a final concentration of 250 µmol/l of various nucleotides. Results are depicted in table 2 and indicate that ATP is about 3 times more stable than dATP and 15 times more stable than all other deoxynucleotides. Therefore, we consider ATP to be the most suitable substrate. Although nucleotides can be stored at −20 °C, repeated freezing and thawing increases the rate of non-enzymatic hydrolysis, making the inclusion of a substrate blank unavoidable.

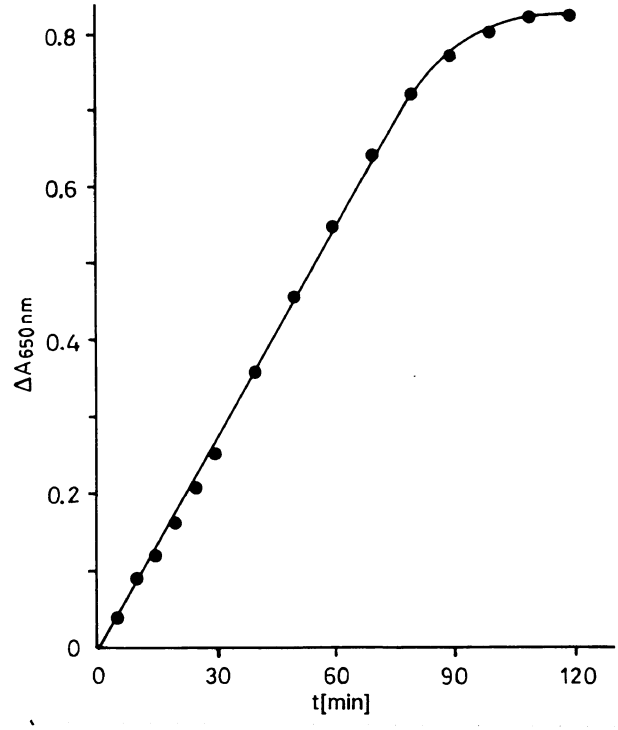

Fig. 3. Linearity of the assay at different incubation times. A pool of 20 sera was incubated with 250 µmol/l dATP at pH 3.0 and 30 °C.

Substrate concentration

As the substrate concentration is increased towards approximate saturation of the enzyme (V_{max}), the substrate blank increases faster than the sample values. It is therefore not possible to determine acid nucleoside triphosphatase activity at an optimal substrate concentration. At ATP concentrations up to 300 µmol/l, the substrate blank does not exceed an absorbance of 0.03. A concentration of 250 µmol/l is recommended as an optimum. This is about 6 times more than the K_m value of ATP, sustaining a rate that is 85% of V_{max}.

Time course of the reaction

As the amount of substrate is the limiting factor of linearity, the time course was monitored at the substrate concentration of 250 µmol/l ATP. Under these conditions the change of absorbance remained linear for 90 minutes (fig. 3).

Linearity and sensitivity

Under standard assay conditions the colorimetric assay was linear to an absorbance of at least 0.8, and sensitive to about 0.3 μmol/l P_i. Sensitivity can be increased by changing the ratio of assay supernatant to colour reagent. Maximum sensitivity is achieved when 500 μl of the supernatant are mixed with 1 ml of colour reagent, but 200 μl of supernatant are usually sufficient.

Influence of anticoagulants

Possible effects of anticoagulants on acid nucleoside triphosphatase activity were assessed under standard assay conditions. No significant differences were found for plasma containing heparin (25 000 U/l), EDTA (1 g/l), citrate (7.6 g/l), fluoride (2 g/l), or oxalate + fluoride (3.4 + 3.4 g/l) when compared with the untreated serum.

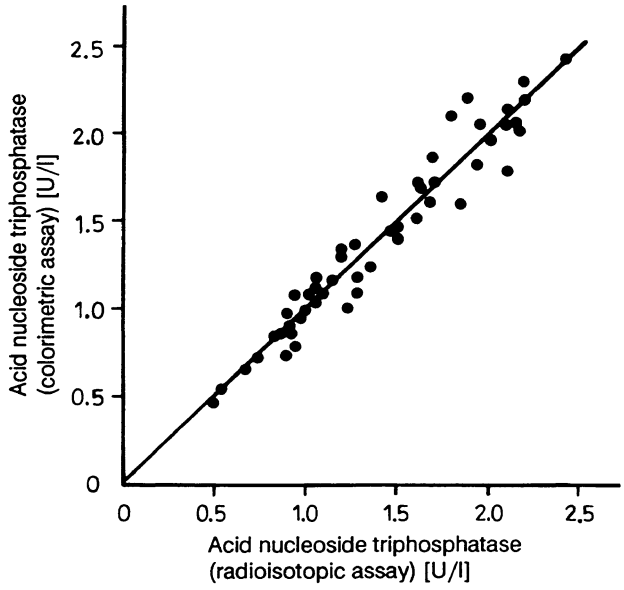

Fig. 4. Comparison of the catalytic activity of acid nucleoside triphosphatase as determined by the colorimetric and by the radioisotopic assay.

Effect of storage

Aliquots of a pool serum were stored at −20, + 4 and + 20 °C. Acid nucleoside triphosphatase activity was determined over a period of 10 days. The data confirm that serum can be stored without loss of activity at −20 °C, + 4 °C, or even room temperature for at least 8 days (data not shown).

Precision

Precision data are listed in table 3.

Tab. 3. Precision data of the assay (n = 20).

Within-series precision			Day-to-day precision		
Mean (U/l)	SD (U/l)	CV (%)	Mean (U/l)	SD (U/l)	CV (%)
1.28	0.033	2.6	0.64	0.025	3.8
2.53	0.060	2.4	2.10	0.097	4.6

Correlation between photometric and radioisotopic test

To evaluate the specificity of the present assay, acid nucleoside triphosphatase activity was determined in 50 sera of healthy blood donors, using the radioisotopic test as reference. Substrate concentration was 60 μmol/l dTTP according to l. c. (1). The correlation found between the two methods was excellent with a correlation coefficient of 0.97 (fig. 4).

Reference values of nucleoside triphosphatase

Nucleoside triphosphatase activity was determined in 100 sera of healthy blood donors (64 men, 36 women) under standard assay conditions with 250 μmol/l ATP as substrate. The mean ± standard deviation was 4.0 ± 1.6 U/l for men, and 3.8 ± 1.9 U/l for women.

Discussion

In order to expand our knowledge of the clinical importance of acid nucleoside triphosphatase, a simple and rapid assay for a large number of patients' samples is required. Before the development of this photometric assay, the enzyme activity had only been measured using a radioisotopic assay involving thin-layer chromatography, which is time-consuming, expensive, and permitting only a few samples to be analysed simultaneously. However, it is easier to determine the enzymic production of inorganic phosphate. A number of methods (6, 7) as well as commercial kits were found unsuitable because of their low sensitivity, whereas the assay methods of *Lanzetta* (5) and *Bencini* (8, 9) are sensitive enough for the estimation of a P_i concentration in the micromolar range. The method developed by *Bencini*, however, uses short wavelengths, and therefore the presence of proteins interferes with the assay. Other assay methods for determining inorganic phosphate are unsuitable if labile phosphate esters such as nucleoside triphosphates are present in high concentrations (10).

The present photometric method is simple, economical and a large number of samples can be analysed within a few hours. Its specificity has been proved in comparison with the radioisotopic assay, which measures the specific reaction product, nucleoside diphosphate. Precision is excellent, with extensive linearity.

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